Vanadium containing Bromoperoxidase – Insights into the enzymatic mechanism using X-ray crystallography

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Abstract

The structure of the vanadium bromoperoxidase from the red algae *Corallina pilulifera* has been solved in the presence of the substrates, phenol red and phloroglucinol. The results obtained give a putative location close to the active site of the enzyme. In addition bromide has been soaked into the crystals and has been shown to bind unambiguously within the active site using single anomalous dispersion. A mutant of the enzyme where arginine 397 has been changed to tryptophan, shows a different behaviour on bromide binding regarding the displacement of a specific leucine amino acid which is seen to move towards the incoming ion in the wild-type protein to produce a hydrophobic environment within the active site.

These results have increased our understanding of the mechanism of the vanadium bromoperoxidases and have demonstrated that the substrate and bromide are specifically bound to the enzyme active site.

Introduction

The vanadium bromoperoxidase enzymes has attracted much interest in recent years due to their applications in biocatalysis for the introduction of bromine groups to aromatic compounds many of which have anti-bacterial and anti-cancer properties. They have also fascinated inorganic chemists and biochemists with respect to their mechanistic details and dependence on vanadium for their activity.

The vanadium bromoperoxidase enzymes from two members of the red algal species, *Corallina*, have been previously studied in detail at a structural level [1,2]. These enzymes form a large dodecameric structure with 12 protein subunits each containing vanadium V which is essential for activity. The structure of *C. officinalis* vanadium haloperoxidase was solved to 2.3Å in 2000 by Isupov *et al*., [1] and showed a 595 amino acid chain folded into a single α + β type domain. The structure does not contain any disulfide bridges as found in the related *Ascophyllum* bromoperoxidase [3]. There are twelve monomers, each consisting of 19 α-helices which are 6 to 26 residues in length, eight 3_{10} helices and 14 β-strands which are mainly involved in β-hairpins. One surface of the monomer is flat and upon dimerisation this surface forms the central region resulting in two four-helical bundles at the centre of each dimer. The active site cleft uses amino acid residues from both monomers, with the residues of one predominantly being responsible for the bottom of the active site binding the vanadate, while the other constitutes the top region of the active site cleft . The dimers then interact to form the dodecamer with a 23 cubic point symmetry, which is approximately 150Å in diameter [1] (Figure 1). Two vanadate atoms are associated with each dimeric subunit and are co-ordinated in a trigonal bipyramidal geometry with hydroxide and His496 in axial positions and three non-protein (solvent) oxygen atoms in equatorial positions. The original structure, due to the availability of crystals

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suitable for the X-ray structural solution, was solved with phosphate in place of the vanadate in the active site of the enzyme this was due to the high concentration of phosphate used in the crystallisation conditions. Phosphate prevents vanadate binding and it has been by others in the study of a fungal vanadium chloroperoxidase if phosphate binds to the active site phosphatase activity can be observed [4,5]. The structure of the vanadate bound form of the *Corallina* vanadium bromoperoxidase has now been solved using a different crystal form of the enzyme grown from polyethylene glycol in the presence of potassium bromide (KBr) [2]. The structure of the enzyme's active site does not show any significant difference to that of the phosphate-complexed structure, other than the coordination of vanadate with His553.

The *Corallina* vanadium bromoperoxidase is found to be stabilised by calcium and vanadium bound to the enzyme. One divalent cation (Ca^{2+} or Mg^{2+}) is observed per monomer from the crystallographic analysis. A systematic study regarding the role of metal ions on the stability of the enzyme has been carried out [6]. The structural analysis of the *C. pilulifera* recombinant enzyme over-expressed in yeast revealed that it contains one calcium atom per subunit which is found in the loop between the amino acid residues at 359 and 366, and that it is coordinated by the main chain oxygen atoms of Phe359, Gln361 and Gln368 and the carboxyl groups of Asp363 and Asp366 and a molecule of water [2].

Crystallographic studies have provided some information to help understand the mechanism of the *Corallina* vanadium dependent haloperoxidases. The nature of the brominating agent for this enzyme has been described as a 'Br^+-like intermediate' being either a free halogenating agent such as HOBr, Br_2, or Br_3^- or an Enz-Br or V_{enz}-OBr species [7]. EXAS studies [8] have shown that the bromide binds within the active site in proximity to the vanadate centre. This would permit the bromide to

directly attack the peroxyvanadate centre in the first step of the halide oxidation in the enzyme mechanism.

With knowledge of the overall structure of the dodecameric *Corallina* bromoperoxidase enzyme an active mutant dimeric enzyme has been made where a substantial portion of the N-terminus of each monomer has been deleted [9]. This enzyme can be over-expressed in *Escherichia coli* in the soluble fraction whereas the dodecameric form is only overexpressed as inclusion bodies. However it has also been possible to refold the recombinant native form of the enzyme *in vitro* to produce an active dodecameric form that resembles the wild type enzyme isolated from the *Corallina* species [10].

The structural information previously carried out has allowed an understanding of the halide specificity of the *Corallina* bromoperoxidase and a mutant enzyme has been constructed of the *C. pilulifera* bromoperoxidase which also has chloroperoxidase activity [11]. The study presented in this paper presents the structural determination of this mutant enzyme.

The structure of wild-type recombinant *C. pilulifera* bromoperoxidase has been solved in the presence of the substrates, phenol red and phloroglucinol. In addition bromide has been soaked into the wild-type and mutant enzyme crystals which has allowed its location within the active site of the enzymes.

2. Experimental

*2.1-Purification of Recombinant and Mutant Enzymes-*The enzymes were cloned and over-expressed and assayed for activity as previously described [2, 6, 11,12].

*2.2- Crystallisation-*The crystallisation trials with the recombinant wild-type enzyme were set up using both hanging and sitting drop vapour diffusion techniques based on previously reported conditions [13,14]. Two crystal forms were obtained, one using ammonium phosphate as precipitant, an hexagonal form, and the second a cubic

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vanadium bound called form 2. The best crystals of form 2 which were only produced

with the wild-type enzyme were using an initial protein of 18mg/ml in 50mM Tris-

H_2SO_4, 0.4M KBr, 1mM Na_3VO_4, 20% (w/v) polyethylene glycol 6000. The wild-type

enzyme was also crystallised in form 1 as previously described [14]. The mutant enzyme

could only be crystallised in form 1 under conditions of 100mM Tris-HBr pH 7.4, 1.3M

ammonium phosphate giving a final pH of 4.0. A cryoprotectant at 100mM Tris- H_2SO_4,

pH 8.5, 1.6M ammonium phosphate, 25% (v/v) glycerol was used for data collection.

For the wild-type enzyme crystals grown from polyethylene glycol 6000 a mother liquor

substituting the precipitant with 25% polyethylene glycol 400 and 25% polyethylene

glycol 6000 was used to allow cryo-cooling without formation of ice. The crystal

soaking experiments were carried out with a 5% addition of phenol red and

phoroglucinol to the relevant cryoprotectant. The bromide soaks were carried out by

making the cryoprotectant 50mM potassium bromide. Soaks were carried out for a

variety of times from 30seconds to several minutes and monitored by visual examination

of the crystals for cracking etc.before freezing for data collection.

2.3- *Data Collection-*

All data was collected under cryocooled conditions at the Hamburg Synchrotron

Facilities, DESY, on Station BW7a. The beamline wavelength was adjusted to 0.9076Å,

close to the absorption edge of the element bromine (0.9202Å) in order to use the

anomalous dispersion to locate the potential bromine ions in the structure. The

crystallographic data was processed and scaled using the HKL package [15]. Molecular

Replacement procedures were carried out using the program AmoRe from the CCP4

package [16]. The refinement procedure was carried out using REFMAC [17] with

graphical support of the programs O [18] and COOT [19]. The analysis of the anomalous

signal was performed using the programs fft and peakmax CCP4 [20]. Libraries with the

chemical and geometric descriptors for chemically modified residues of the mutant protein were created using the program Sketcher and minimisation calculations performed with the program REFMAC, CCP4i graphical interface. The molecules of water within the structures were located and analysed with the program Woda (Teplyakov, personal communication). Analysis of the quality of the data and the structure solutions were carried out using the programs SFCHECK [21] and PROCHECK [22].

3. Results

We have used *Corallina* vanadium haloperoxidase enzymes and a selected mutant, arginine397tryptophan that have been crystallised and subjected to substrate soaking experiments with phenol red, phoroglucinol and potassium bromide. The substrate binding sites have been proposed to bind to a hydrophobic area in the active site channel of the enzyme. The level of occupancy of the substrates does not allow detailed interactions to be seen.

The addition of bromide to the crystals has produced unambiguous evidence for the binding of the halogen in the active site of the enzyme. The analysis of the anomalous signal has allowed the location of the bromide in front of the arginine 397 residue in the wild-type enzyme cubic crystal form. The bromide is positioned within hydrogen bonding distance (2.8 -3.1 Å) between the vanadate ion and the arginine residue as shown in Figure 2 in both cartoon mode and with the electron density. There was also an anomalous scatterer located on the solvent exposed surface of the enzyme within covalent bonding distance to two tyrosine residues, Tyr186 and Tyr43. The bromination which is weak occurs on the (o-) position to the hyroxyl group and the residue appears to be mono-halogenated. It was also possible to locate the binding of bromide in the mutant enzyme hexagonal crystal although the position of the halogen is slightly different. As a

result of the absence of Arg397 the bromide is missing the hydrogen-binding interaction that held it in the active site of the wild-type enzyme. Instead it is located very close (3.0 Å) to one of the phosphate oxygen atoms and in front of the newly introduced tryptophan residue. The difference between the two halogen positions is 2.5 Å. The bromination of the tyrosine residues described above is also observed for the mutant enzyme.

Examination of the electron density after refinement of the structures results in a significant change of position of Leu337 in the wild-type enzyme towards the inner active site. This change suggests a relation of this movement with the presence of the halogen in the active site. This was not observed with the mutant enzyme (Figure 3). An additional change on bromide binding was the rotation by 25° of a phenylalanine residue, Phe373, which is between the two monomers that make up the catalytic dimer.

 In the non-halide bound structure the position in which the halogen and the Leu337 side chain are located is occupied by one molecule of water in the equivalent position. When the halide is bound it appears that there is a formation of a hydrophobic pocket accompanied by a reduction in the size of the entrance to the entrance of the active site as shown in Figure 4. This eliminated water from the active site providing a hydrophobic environment for the reaction to occur. The crystal data sets of these halide complexes have been collected to a resolution of 1.78-2.5Å. and the statistics of the data are given in Table 1 and 2. The structural information will be deposited in the PDB with codes (XXX and XXX).

4. Discussion

For many years it had been questioned as to whether the haloperoxidases bind their substrates, or whether they catalysed only the formation of the brominating agent and allowed the reaction to occur non-specifically. It has been demonstrated for the

bromoperoxidase from *A. nodosum* that there is a competition between indole and phenol red bromination [23]. In addition this group also demonstrated that the indole was binding to the active site as evidenced by fluorescence quenching. Furthermore, the same enzyme will catalyse a regiospecific indole bromination [24]. These results again are direct evidence that the reaction is occurring within the enzyme catalytic site. We have used *Corallina* vanadium haloperoxidase enzymes and a selected mutant, arginine397tryptophan that have been crystallised and subjected to substrate soaking experiments with phenol red and phoroglucinol. These substrate binding sites have been located to a hydrophobic area in the active site channel of the enzyme. The *Corallina* bromoperoxidase enzyme shows a remarkable electronegative surface with the exception of the depth of the active site of each monomer where it exhibits a strong electropositive potential. Other slightly electropositive areas observed in the figure are burioed when the dimers adopt their dedodecameric arrangement. This difference in electrostatic potential allows the substrates to directly locate the active site of the enzyme. The upper part of active site channel of the enzyme is lined by hydrophobic residues on one side and hydrophilic on the other side. It has previously been proposed [3] that this could be involved in binding of organic substrates. It is on this basis that a putative substrate binding site has been proposed from the limited data obtained in this study.

In addition crystallographic studies on the native and mutant protein have also been carried out by both co-crystallisation and soaking experiments with potassium bromide. This has shown that there is a specific halogen-binding site involving Arg397 in the enzyme and that there is involvement of the surrounding amino acids. All of the data sets in which halogen has been introduced show a consistent anomalous peak at approximately 3.6Å from the vanadate (01), which is consistent

with a previous proposal [8]. The halogen atom appears to be bound to one of the terminal nitrogen atoms of Arg397 at a distance of 3 Å. The remaining residues in the environment of the bromide ion are mainly hydrophobic that is a common feature of halogen binding to proteins [25]. The presence of bromide in the active site is associated with the displacement of Leu337 to form the hydrophobic pocket. This movement has not previously been observed. The environment of the active site could further change upon binding of peroxide but as yet no peroxo-complex has been crystallised.

The activation of the *Corallina* bromoperoxidase enzyme towards the vanadate and the formation of the peroxovanadate intermediate is carried through different finely tuned mechanisms. The binding histidine residue His553 has strong several strong interactions via one of the nitrogen atoms of the imidazole ring which converts the remaining nitrogen into a highly reactive position and possibly changing the pKa of this residue. The vanadate ion is held in co-ordinating distance of this histidine by several hydrogen bonding interactions with the surrounding amino acids. Of these interactions the strongest is between Lys400 (2.4 Å), followed by vanadate (03) to Ser485 (2.7 Å). Lys400 together with Arg547 and Arg408 establish a protonated network around the vanadate ion [26]. These residues displace the charge on the vanadate ion, elongating the bonding distances between the equatorial oxygens and the vanadate and activating the ion towards attack by the hydrogen peroxide. The interaction and the role of His487 is relatively weak in bromination [26]

The studies described in this paper have lead to an increased understanding of the structure of these bromoperoxidase enzymes and the role of the vanadium in the mechanism which is essential for this enzymatic bromination reaction.

Acknowledgements

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	Wild-Type form 1	Wild-Type form 2	Mutant form 1
Space group	P63	P 21 3	P63
Unit cell a/b/c Å	184.01, 184.01,177.93	199.60, 199.60,199.60	182.48, 182.48, 177.59
Resolution range (Å)	15.00-2.38	14.92-2.15	29.87-1.78
No. measured reflections	116933	141422	307595
No. unique reflections	116933	141422	307595
Completeness	86.0%	99.2%	96.3%
Final R_{cryst}	0.223	0.152	0.163
R_{free}	0.28	0.22	0.19
Average B-factor (protein) (Å^2)	33.1	27.1	22.2
Average B-factor (solvent) (Å^2)			
Rms deviation from ideality			
Bond lengths (Å)			
Bond angles (°)			

Table 1

Figure Legends

Figure 1

The dodecameric structure of the *Corallina* vanadium bromoperoxidase. Each subunit is shown in a different colour.

Figure 2

The location of the bromide in the wild-type *Corallina* vanadium bromoperoxidase from the data set of crystals of form 2 in the presence of KBr and vanadate. The bromide is shown as a cyan sphere bound between the vanadate and residue Arg397 at an H-bonding distance of 2.8-3.1Å. in cartoon mode. Both subunits are needed to create the active site pocket. Figure created with PYMOL [27].

Figure 3

A figure showing the superimposition of the active site residues of the wild-type *Corallina* vanadium bromoperoxidase crystal form 2, bromide bound (green backbone) and wild-type *Corallina* vanadium bromoperoxidase crystal form 1, non-bound (yellow backbone) structures. The displacement of Leu 337 and Phe373 is indicated with arrows. The figure is prepared with the program COOT [19].
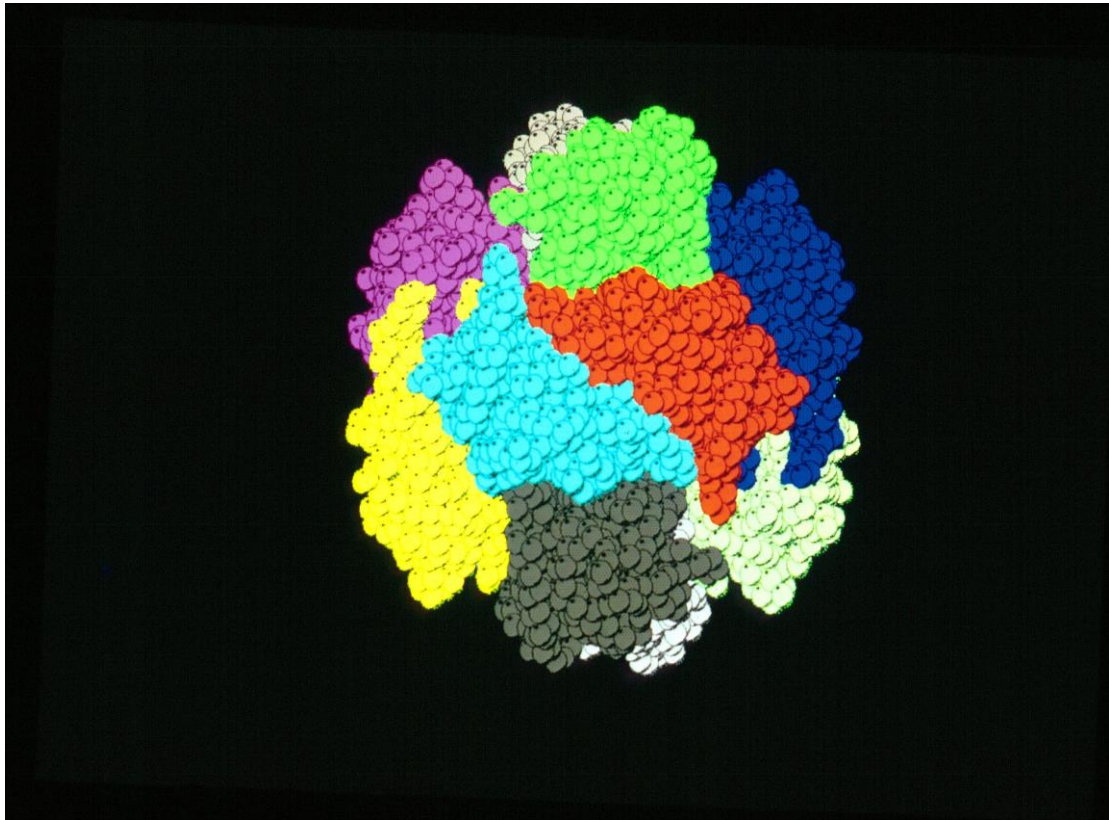
Figure 4

The electron density for the phosphate ion, His553, Trp397 and Leu337 (pink) pointing away from the active site in the mutant Arg398Trp *Corallina* vanadium bromoperoxidase enzyme. The bromide ion is shown as a purple sphere and the Cα backbone in green ribbon. The maps are drawn at one sigma (2Fo-Fc) and the positive difference map (in blue) at three sigma (Fo-Fc). The figure was prepared with the program BOBSCRIPT [28].

Figure 5

A surface representation of the active site of the wild-type *Corallina* vanadium bromoperoxidase in the absence (above) and presence (below) of the halogen (cyan sphere) showing the reduction in entrance of the active site between the crystal form one and the crystal form 2, vanadate, bromide bound structures. The green surface and side chains represent hydrophobic residues and the yellow non-hydrophobic residues.

The surface was calculated with the MSMS package [29] and rendered with CHIMERA [30].
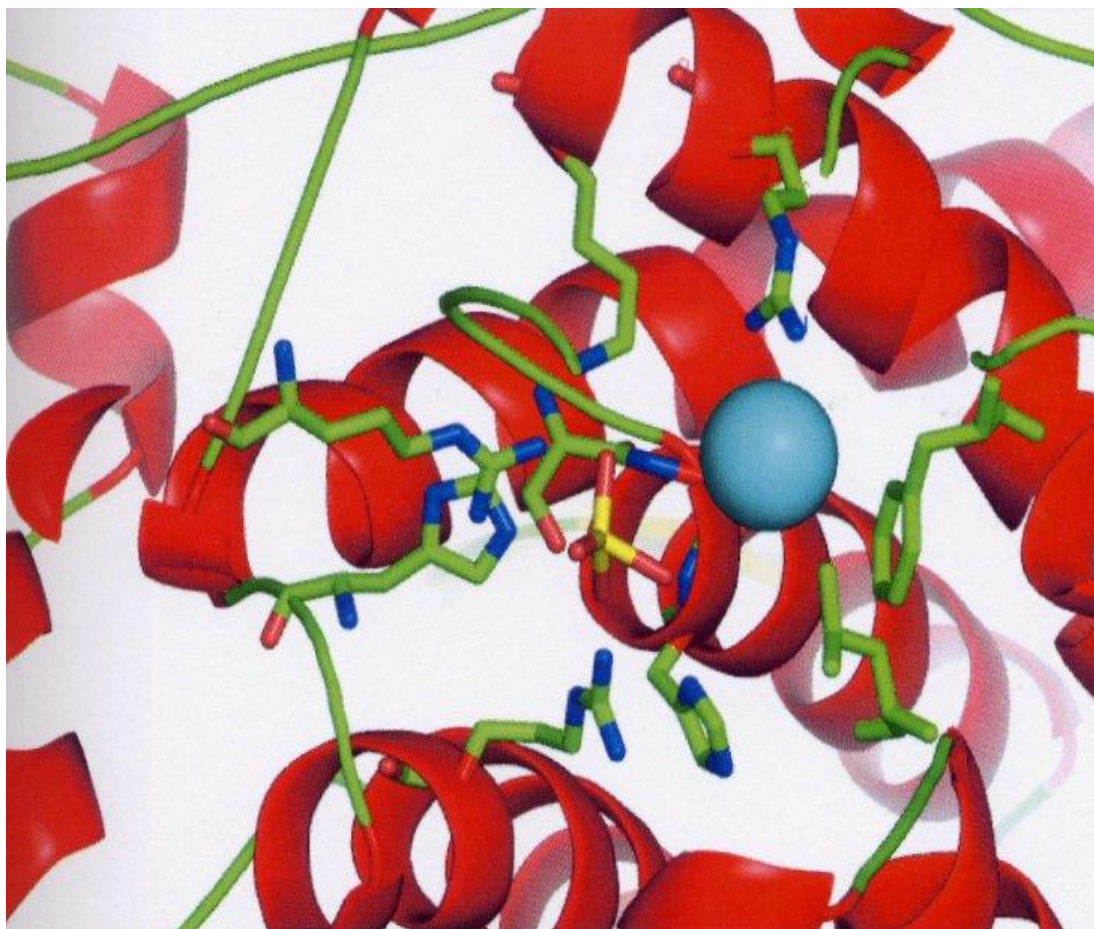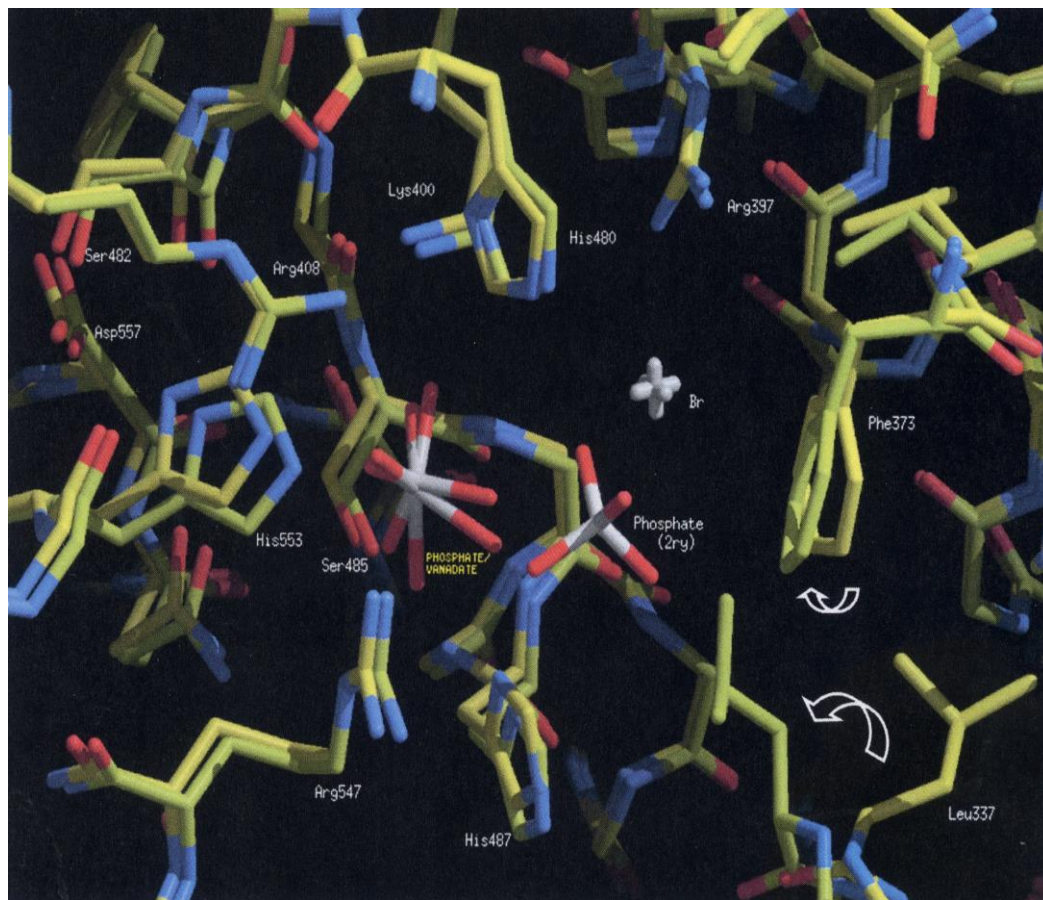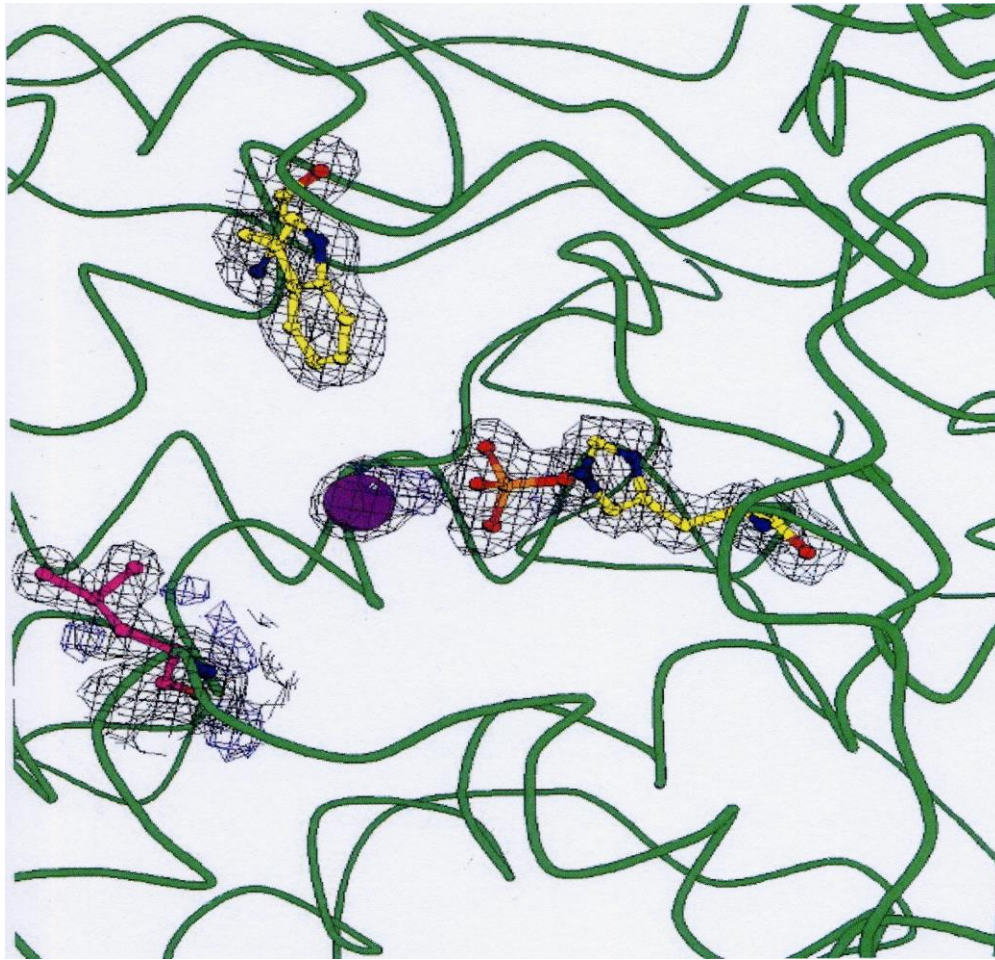
Figure1

Figure 2

Figure3

Figure 4

Figure 5